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The Metabolism of Tri-o-cresyl phosphate in Cats

by



Norman Wagner

A Thesis

Submitted to the Faculty of Graduate Studies in Partial

Fulfilment of the Requirements for the

Degree of Master of Science

Department of Pharmacology

Edmonton, Alberta, 1968



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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Metabolism of Tri-o-cresyl phosphate" submitted by Norman Wagner in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

This investigation was undertaken to determine whether tri-ortho-cresyl phosphate (TOCP), a tri-aryl phosphate ester, could be hydrolyzed by various phosphatases in vitro and thus provide evidence for a step in TOCP metabolism. Calf mucosal alkaline phosphatase, potato acid phosphatase and brussel sprout phosphodiesterase were used to attempt the hydrolysis of TOCP. The substrates used to determine whether these enzymes were active were disodium phenyl phosphate and sodium diphenyl phosphate. Phosphatase activity was assessed by measuring the phenol liberated during hydrolysis (Wooton, 1964). Neither TOCP nor triphenyl phosphate (TPP) were hydrolyzed by these enzymes.

In addition, we investigated the possibility that TOCP might be metabolized to o-hydroxybenzyl alcohol and/or o-cresol with subsequent conversion to salicylate. Urinary salicylate was assayed after the conversion to salicylate. Urinary salicylate was assayed after the intraperitoneal administration of 100 mg/kg TOCP to cats.

After the administration of o-cresol, o-hydroxybenzyl alcohol and sodium salicylate to different cats, the urine from each cat was assayed for salicylate and for the compound administered. The salicylate assay was carried out using a fluorometric method (Aidie, 1967). The identification of o-hydroxybenzyl alcohol and o-cresol in urine was accomplished with the aid of a Microtech gas chromatograph. Our results showed that these possible metabolites of TOCP can all be assayed in the urine as salicylate.

After the intraperitoneal administration of TOCP-³H to three cats, urine was collected for 8 consecutive days. The urine was subjected to salicylate assay, assay for tritium in a liquid, scintillation counter, and gas chromatography. Although small amounts of TOCP-³H were excreted in the urine, no significant increase in salicylate was found. These results indicated that the tissues of the cat act as a reservoir for TOCP and only minute amounts of 2-(o-cresyl)-4:1,3,2-benzodioxaphosphoran-2-one (CBDP) may be formed in this species, or that the rate of transformation of TOCP to CBDP is very slow.

DEDICATION

I dedicate this thesis to my loving wife

LINDA

and to my parents,

CHARLES and MIRIUM

CECIL and MENA

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to Dr. Jack Dean Taylor who has assisted and guided me in all ways including the writing of this thesis as well as other learning experiences of the past years.

In addition, I would like to thank Corine Fincham whose presence always brightened up our laboratory, and whose hands always did my dishes.

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TABLE OF CONTENTS

	PAGE
Abstract	i
Acknowledgements	iii
List of tables	iv
List of figures	v
Introduction	1
Statement of problem	7
Methods and Materials	8
A. Enzyme preparations and reagents	8
B. Assay of enzyme activity	10
1. Potato acid phosphatase	10
2. Alkaline phosphatase	10
3. Brussel sprout phosphodiesterase	11
C. Chromatography	12
1. Adsorption column chromatography	12
2. Paper chromatography	12
3. Gas chromatography	13
D. Ultraviolet spectrophotometry	14
E. Detection of radioactivity	14
F. Determination of salicylate (Aidie, 1967)	16
G. Modified method for the determination of salicylate	17
H. Collection of cat urine	20
I. The attempted recovery of salicylate from cat urine following oral administration of sodium salicylate, or o-hydroxybenzyl alcohol and intraperitoneal administration of o-cresol	20

	PAGE
J. Efficiency of the recovery of TOCP, o-hydroxybenzyl alcohol, and o-cresol added to cat urine.	22
K. Attempted recovery of o-hydroxybenzyl alcohol from cat urine following the administration of o-hydroxybenzyl alcohol and o-cresol.	23
L. Attempted recovery of o-cresol from cat urine following the intraperitoneal administration of o-cresol.	24
M. Tri-o-cresyl phosphate and its metabolites.	25
Results	26
1. Assay of enzyme activity	26
a. acid phosphatase	26
b. alkaline phosphatase	26
c. phosphodiesterase	26
2. Recovery of salicylate from cat urine after its oral administration.	29
3. Recovery of salicylate in cat urine after oral administration of o-hydroxybenzyl alcohol.	29
4. Recovery of o-hydroxybenzyl alcohol after the oral administration of o-hydroxybenzyl alcohol and intraperitoneal administration of o-cresol.	30
5. Recovery of o-cresol and salicylate from cat urine following the intraperitoneal administration of o-cresol.	32
a. test for phenols	32
b. assay for o-cresol	32
c. assay for salicylate	34
6. Column chromatography	39

List of Tables

1. Conditions for gas chromatography of o-cresol, TOCP,
and o-hydroxybenzyl alcohol. 13
2. Summary of the efficiency of the recovery of TOCP,
o-hydroxybenzyl alcohol, and o-cresol added to cat urine..... 28
3. Assay of cat urine for TOCP and tritium following
the administration of TOCP-³H to cats. 42a
4. Amount of tritium administered to cats. 43a
5. Summary of the results obtained for the metabolism and
excretion of TOCP, salicylate, o-cresol, and o-hydroxy-
benzyl alcohol. 44

List of Figures

1. A proposed metabolic pathway of TOCP	4
2. Absorption spectrum of TOCP	15
3. Method of Aidie (1967)	18
4. Modified method of Aidie (1968)	19
5. Cage for urine collection	21
6. pH-activity curve for potato acid type II phosphatase	27
7. Standard curve for salicylate assay (Aidie, 1967)	31
8. Standard curve for o-hydroxybenzyl alcohol using a Microtech gas chromatograph	33
9. Standard curve for o-cresol in cat urine (Folin-Ciocolteau).	35
10. Amount of phenol excreted in cat urine after administra- tion of o-cresol to cats	36
11. Standard curve for o-cresol using a Microtech gas chromatograph	37
12. Standard curve for salicylate assay using the modified method of Aidie (1968).	38
13. Adsorption column chromatography of TOCP- ³ H.	40
14. Radioactivity assayed in cat urine after the administration of TOCP- ³ H.	43
15. Standard curve for TOCP using a Microtech gas chromatograph	44a

Introduction

Considerable interest has been attached to the metabolism of tri-o-cresyl phosphate (TOCP) ever since its toxic effects in man were realized during the prohibition era in the United States. Illegal beverages containing TOCP produced, in man, a flaccid paralysis of the extremities. Subsequent investigations on the toxicity of TOCP in several species of animals and in chickens (Smith et al, 1931, 1932) have shown that TOCP is capable of producing ataxia, paralysis, and eventual nerve demyelination.

Smith & Stohlman (1934) have shown that after the intravenous administration of TOCP to rats, rabbits, and cats, approximately 85% of the TOCP could be recovered from the lungs after several days. In addition, small amounts of TOCP were recovered from the urine of these species. Gross and Grosse (1932) have shown that after the intraperitoneal administration of TOCP to rabbits, a large proportion of the administered tri-cresyl ester could be recovered from the intestine and liver.

It was later shown by Mendal and Meyers (1953) and Aldridge (1954) that tri-o-cresyl phosphate exhibits little inhibitory activity against esterases in vitro but can cause cholinesterase inhibition in vivo. Meyers et al. (1955) found an active esterase inhibitor in the urine of rats and rabbits after the administration of TOCP to these animals. These findings suggested to these workers that the cresol esters measured by Gross & Grosse (1932) might in fact be

active derivatives of TOCP. They subsequently assayed the tissues and urine of rats for esterase inhibiting activity after the administration of TOCP to these rodents. Their results showed that an active inhibitor of pseudocholinesterase was extracted which was 3,000 to 6,000 times more potent than TOCP. In addition, they found that approximately 80% of the total esterase inhibitor activity was located in the intestinal tract while 12% was recovered from the liver and 8% from the remainder of the body. Meyers et al. (1955) have also shown that a similar inhibitor of pseudocholinesterase can be recovered from the liver and intestine of rats after the administration of triphenyl phosphate. Since Block (1943) had previously reported that esterases of the rabbit can be inhibited in vivo after the administration of tri-o-chlorophenyl phosphate as well as by TOCP, Meyers et al. (1955) proposed that the primary alteration in the structure of the triaryl phosphates leading to the formation of active inhibitors in vivo is a substitution on one or more of the phenyl rings.

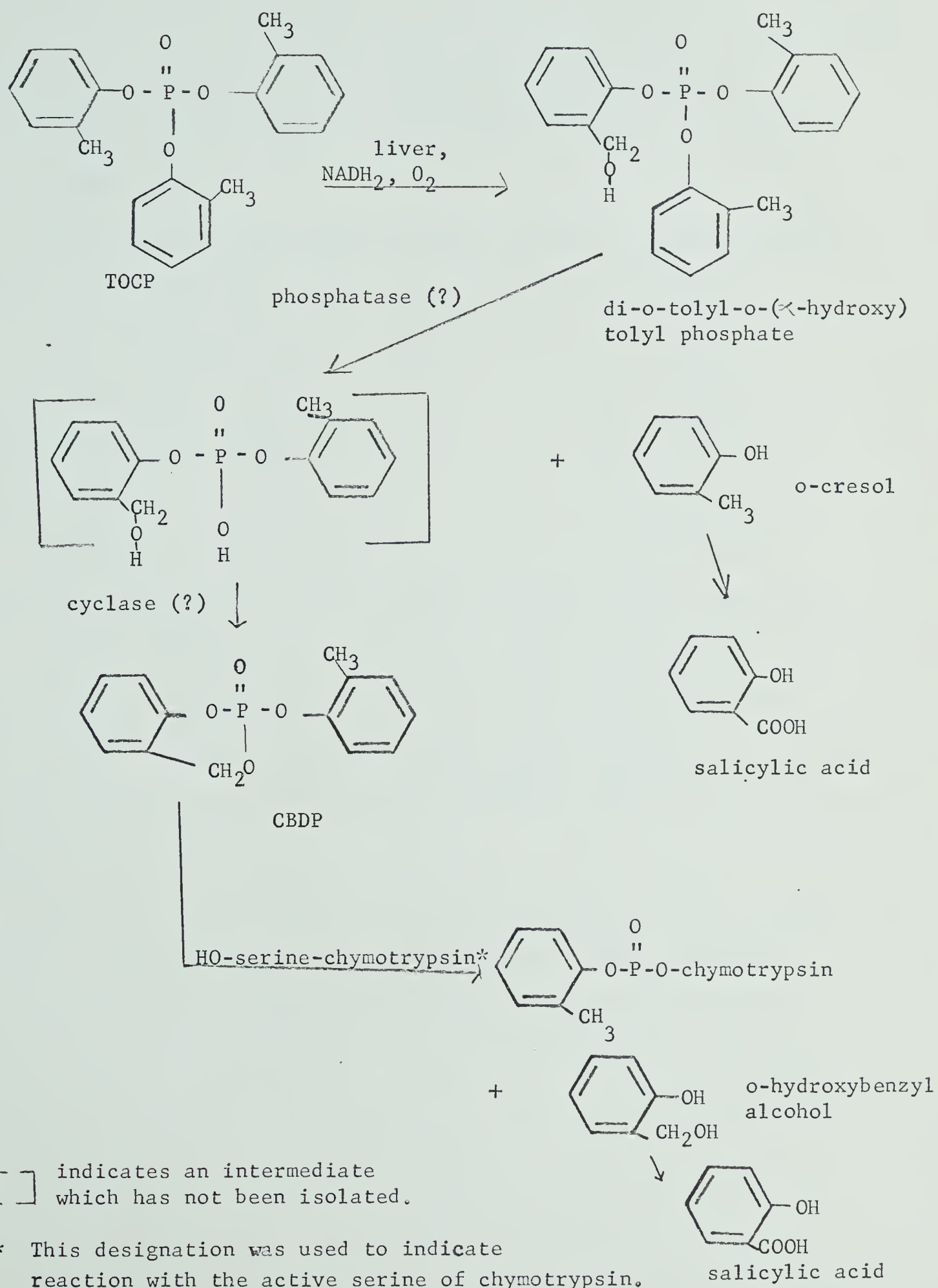
An interesting study in the fowl by Hine et al. (1956) on the relationship between various members of a series of substituted phenyl phosphates and their ability to produce neurotoxic paralysis and cholinesterase inhibition showed that these aromatic phosphates which contained one or more orthotolyl groups, with the exception of triorthotolyl borate, produced paralysis in chickens. In addition, all the above compounds inhibited, in vivo, fowl plasma cholinesterases, but none affected fowl brain cholinesterases severely.

It was ultimately demonstrated by Eto, Casida, and Eto (1962), after isolation from rat liver and intestine of 2-(o-cresyl)-4:1,3,2-benzodioxaphosphoran-2-one, hereafter designated CBDP, that TOCP is indeed metabolized to an active metabolite. These workers demonstrated that synthetic CBDP inhibited α -chymotrypsin and yielded o-hydroxybenzyl alcohol. Presumably CBDP could react with other enzymes which contain an active serine, in fact, CBDP is known to be a cholinesterase inhibitor. It was subsequently shown that synthetic CBDP could produce ataxia, and nerve axon degeneration in cats (Taylor, 1967) and hens (Casida, Eto, and Baron, 1961) similar to the toxicity produced by TOCP.

Eto & Casida (1967) demonstrated in vitro that TOCP, in the presence of rat liver homogenate, NADH_2 , and O_2 , is oxidized to yield di-o-tolyl-o-(o-hydroxy) tolyl phosphate which was subsequently shown to be cyclized and hydrolyzed by plasma albumin to yield the active metabolite CBDP.

Figure 1

A proposed metabolic pathway of TOCP



In the conversion of TOCP to CBDP in vivo, o-cresol, o-hydroxybenzyl alcohol, and sodium salicylate are possible metabolic products (Eto & Casida, 1962). However, these compounds have not been identified after the administration of TOCP. This work was focused on the isolation and identification of these possible metabolites of TOCP in cat urine.

Cats were used because CBDP has been isolated from cat intestine (Buttar, 1967), and high levels of phenols have been reported in cat urine (Gross and Grosse, 1932) after the administration of TOCP. Smith et al. (1934) were unable to detect an increase in blood phenols following intravenous TOCP administration. They suggested that the phenols reported by Gross & Grosse resulted from hydrolysis of TOCP.

In addition, we were unable to find well documented examples of enzyme hydrolysis of tri-aryl phosphates such as TOCP. Since TOCP is a tri-aryl phosphate ester, we thought that hydrolysis with a phosphatase may be a step in the metabolism of TOCP. The work of Eto et al. (1961), in rats, in which mono and diaryl phosphates were isolated in the urine after the administration of TOCP, indicated the existence of such a phosphatase.

Eto et al. (1962), suggested that the metabolism of TOCP in vivo leading to the formation of CBDP and subsequent enzyme inhibition should also yield o-cresol, o-hydroxybenzyl alcohol and salicylate.

Although the toxicity of o-cresol (Deichmann, 1944) and sodium salicylate (Waddell, 1911) have been investigated in cats, studies on the metabolism and excretion of these compounds are lacking for this species. Neither the toxicity nor metabolism of o-hydroxybenzyl alcohol has been studied in cats.

Previous studies in the rabbit indicated that approximately 98% of the administered salicylate can be recovered as the free or conjugated salt (Williams, 1959) and it was found that approximately 85% of the material was recovered in the urine primarily as a conjugate with glucuronide and about 2% of the administered o-cresol appeared in the urine as gentisic acid. Although salicylic acid is a possible metabolite of o-cresol, salicylic acid has not been previously found after the administration of o-cresol to animals.

o-Hydroxybenzyl alcohol has been shown to be metabolized to salicylic acid in rabbits (Williams, 1938) and a significant amount of the alcohol appears in the urine as a conjugate with sulphate or glucuronide. Little information on the metabolism of this compound in other animals is available.

STATEMENT of the PROBLEM

The present work undertaken to study the metabolism of TOCP in cats. Initially we sought to determine whether an alkaline phosphatase, an acid phosphatase, or a phosphodiesterase can hydrolyze TOCP in vitro.

In addition, the metabolic fates of sodium salicylate, o-hydroxybenzyl alcohol, and o-cresol were investigated after their administration to cats. Since any of these compounds, or their metabolites, are themselves possible metabolites of TOCP, and because no previous investigations regarding their metabolism or excretion in this species had been reported, we felt that this investigation was necessary.

Finally, a urinary salicylate assay was performed after the administration of TOCP-³H to cats in order to determine whether o-hydroxybenzyl alcohol and/or o-cresol are formed following the conversion of TOCP to CDBP with subsequent enzyme inhibition.

METHODS and MATERIALS

A. Enzyme Preparations and Reagents.

1. Calf mucosal alkaline type I phosphatase (1 mg/ml).
2. Potato acid type II phosphatase (10 mg/ml).
3. Tween 80.

The reagents listed above were obtained from Mann Research Laboratories, Inc. 136 Liberty Street, N.Y.C., N.Y., 1006.

4. Tri-o-cresyl phosphate, practical grade. This material was purified by vacuum distillation before use because it gradually decomposed when stored at room temperature, liberating phenols.
5. Triphenyl phosphate (TPP). A 0.01% emulsion in 0.01% Tween 80 was prepared.
6. Diphenyl phosphate mono silver salt. The silver salt was first converted into the sodium derivative by the addition of an equimolar amount of sodium chloride. The solution was then centrifuged at 1500 x g for 10 minutes and the supernatant was used as the enzyme substrate. The TOCP, TPP, and diphenyl phosphate mono silver salt were obtained from Eastman Kodak Company, Rochester, New York, U.S.A..
7. Phenyl disodium phosphate was obtained from Fisher Scientific Company, Fair Lawn, New Jersey, U.S.A..

8. M/15 phosphate buffer was prepared using:

a) M/15 Na_2HPO_4

b) M/15 KH_2PO_4

These ingredients were mixed in varying proportions to give the desired pH (Hepler, 1958).

9. Tritiated tri-o-cresyl phosphate (TOCP- ^3H) was prepared by the Wilzbach technique (1957) at our request by New England Nuclear, Boston, Mass.. The radioactive material was purified by adsorption column chromatography before use (Eto et al, 1962) and a 10% emulsion of the purified material was prepared in 10% Tween 80.
10. Salicylic acid, sodium salicylate, o-hydroxybenzyl alcohol, and o-cresol were obtained from Fisher Scientific Company. The o-cresol was purified by vacuum distillation (b.p. 41 c at 14 mm Hg). The material was then stored under nitrogen in sealed glass vials.
11. Scintillation fluid was prepared using 2,5 diphenyl oxazole (PPO, 8 g), 1,4-di-(2-5-phenyloxazolyl)-benzene (POPOP, 200 mg), naphthalene (200 g), made up to 1 liter in dioxane.

B. Assay of enzyme activity:

Each enzyme was assayed in the following manner: The aqueous enzyme preparation (0.5 ml) was incubated at 37⁰ in a water bath with buffer (0.5 ml) and substrate (0.1 ml). The reaction was stopped at the appropriate time by the addition of 0.5N sodium hydroxide (0.8 ml), followed by 0.5N sodium bicarbonate (1.2 ml), 0.5N 4-aminoantipyrine (1.0 ml) and 0.5N potassium ferricyanide (1.0 ml). The solution was allowed to stand for 10 minutes to allow optimum color development. The color development was determined with a Klett-Sommerson colorimeter using a KS 540 filter.

1. Potato acid phosphatase:

The hydrolysis of disodium phenyl phosphate (48 mM) was measured for 15 minutes over a pH range of 5.2 to 6.0 using potato type II acid phosphatase (10 mg/ml) and M/15 phosphate buffer. The pH optimum for this reaction mixture was approximately 5.4. A similar experiment using 0.01% TOCP in 0.01% Tween 80 was also carried out. The reaction was stopped after one hour.

2. Alkaline phosphatase:

The hydrolysis of disodium phenyl phosphate (48 mM) was also measured for 15 minutes over a pH range of 8 to 10 using alkaline type I phosphatase. The buffer was prepared as follows:

- a) sodium carbonate (6.3 g), sodium bicarbonate (3.36 g), were dissolved in water to make 1 liter. The pH of this solution was 10.
- b) M/15 phosphate buffer (described previously). The buffers were mixed in various proportions to give the desired pH. The pH optimum for the alkaline phosphatase activity was approximately nine.

In a similar experiment using 0.01% TOCP in 0.01% Tween 80, no hydrolysis was detected after one hour. A control experiment using 0.01% Tween 80 and disodium phenyl phosphate was performed to demonstrate that Tween 80 did not inhibit hydrolysis.

3. Brussel sprout phosphodiesterase:

The hydrolysis of sodium diphenyl phosphate (200 mM) was measured for 22 hours at a pH of 6.6 using brussel sprout phosphodiesterase M/15 phosphate buffer. Activity of the preparation agreed well with the results obtained from the work of Davidson & Long (1958). In a similar experiment using 0.01% TOCP in 0.01% Tween 80, no hydrolysis was detected after 22 hours. A control experiment using 0.01% Tween 80 and sodium diphenyl phosphate was performed.

C. Chromatography.

1. Adsorption column chromatography.

The column consisted of two parts by weight of silicic acid and one part celite. Both the ingredients were heated to 150^o, cooled to room temperature, slurried in hexane and packed to yield a column. TOCP-³H (1.0 mc) dissolved in benzene (1.0 ml) was diluted with non-tritiated TOCP (5 ml). Benzene (5.0 ml) was added to the mixture. The solution was then pipetted on to the adsorption column. The elution was accomplished with a stepwise gradient of benzene and anhydrous ether. Ten milliliter fractions were collected.

2. Paper chromatography.

Whatman no. 1 filter paper was cut into strips 15-20 cm in length. Salicylic acid was chromatographed by allowing the solvent (20% KCl) to rise about 10-12 cm up the paper. Salicylic acid was detected by its fluorescence using a no. 12 short wave ultraviolet lamp. Approximately 0.1 μ g of salicylic acid could be detected.

3. Gas chromatography

The detection of o-cresol, TOCP, and o-hydroxybenzyl alcohol in cat urine was accomplished using a Microtech gas chromatograph. The column support was a silicone rubber gum SE 30, on a chromport 80/90 column. The chromatograph settings were as shown in table 1.

Table 1

Conditions for gas chromatography of o-Cresol, TOCP, and o-hydroxybenzyl alcohol.

compound	flow (cc/minute)			inlet	temperature (centigrade)		attenuator	
	He	air	H ₂		detector	column	input	output
o-Cresol	8	1.2	6	195	290	125	10	32
TOCP	6	1.2	7	195	290	270	10	16
o-Hydroxybenzyl alcohol	10	1.2	6	195	250	125	10	64

D. Ultraviolet spectrophotometry.

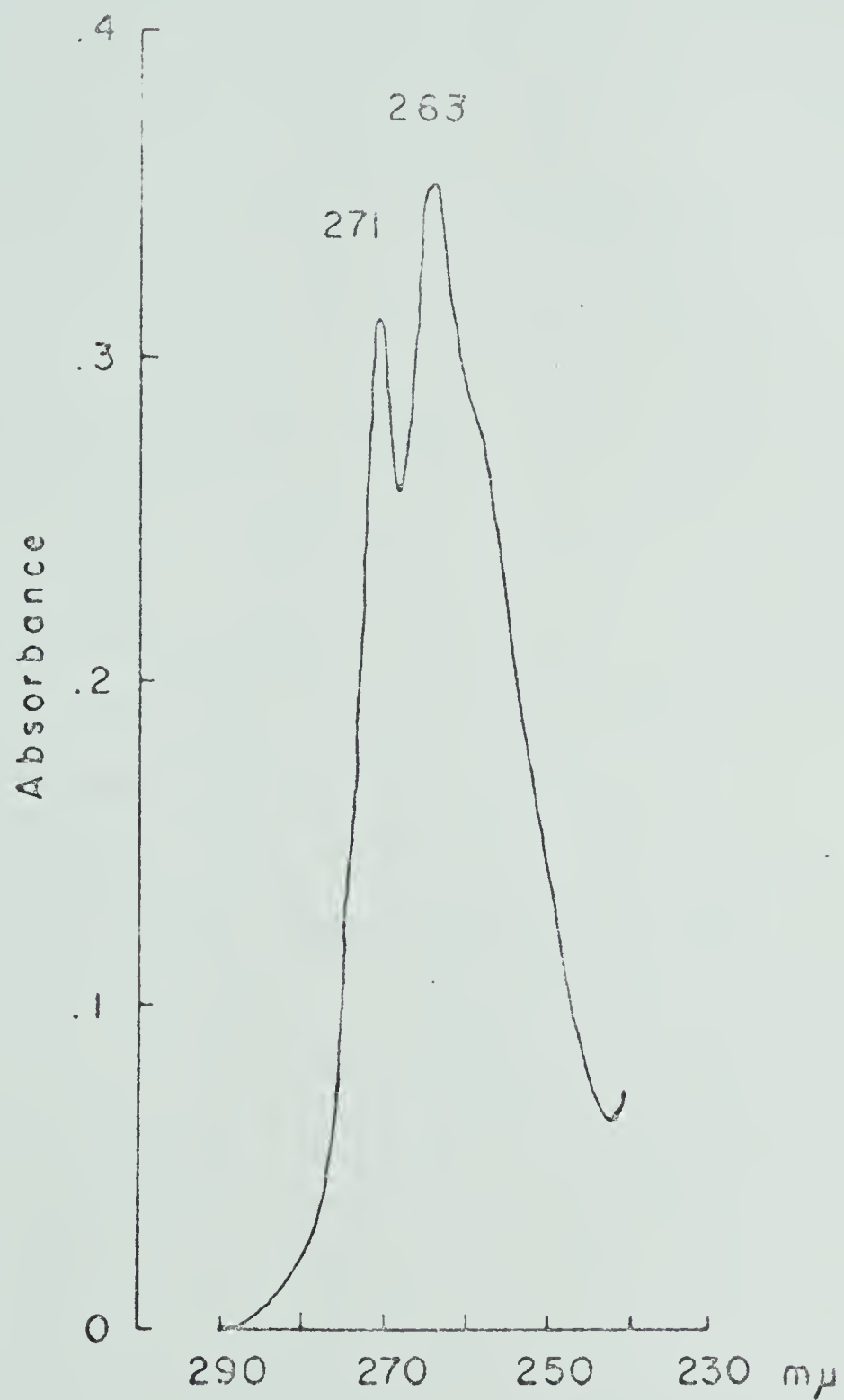
An absorption spectrum of 0.01% TOCP in chloroform was obtained using a Bausch and Lomb recording spectrophotometer. Absorption maxima were observed at 263 nm and 271 nm (figure 2). The Hitachi-Elmer-Perkin spectrophotometer was used to determine the optical density of the fractions obtained from the absorption column. The optical density at 263 nm of the fractions obtained was measured following evaporation of the benzene-ether solvent mixture and addition of chloroform (10 ml). Aliquots were diluted 1/10 with chloroform prior to spectrophotometric assay.

E. Detection of radioactivity.

The Nuclear Chicago model 6850 Unilux Scintillation Counter was used for this determination.

A standard "quench" curve was made based on the channels ratio method using quenched standards. The fractions diluted 1/100 in chloroform were evaporated under nitrogen and redissolved in scintillation fluid (5.0 ml). The vials were then assayed for tritium. The radioactivity was compared with the optical density of each fraction.

figure 2
Absorption Spectrum
of TOCP



F. Determination of salicylate (Aidie, 1967).

The urine sample (25 ml) was diluted with water to a specific gravity of 1.010 and refluxed for one hour with 10N NaOH (2.5 ml). The solution was then cooled and concentrated HCl (5 ml) and phosphotungstic acid (7.5 g) were added. The mixture was cooled in ice water for 15 minutes and centrifuged for 10 minutes at 1500 x g. The supernatant solution was poured into a separating funnel, and 10N NaOH (10 ml) and chloroform (35 ml) were added. The solution was shaken vigorously and the chloroform was discarded. Concentrated HCl (10 ml), and chloroform (15 ml) were added to the aqueous solution. The mixture was shaken vigorously and the chloroform extract was drawn off into a second separating funnel. The salicylate was re-extracted with 1N NaOH (10 ml). The chloroform was discarded and concentrated HCl (2 ml) was added to the aqueous solution. The solution was put through a previously washed ion exchange column consisting of IRA401 resin (0.2 g) in a medicine dropper. The liquid was poured through the resin three times. The column was washed, first with distilled water, followed by 1N NaOH and 0.9% NaCl. The washings were discarded. The salicylate was extracted from the column by elution with 3N NaI (25 ml) which was passed very slowly through the column. 1N HCl (2 ml) was added to the effluent from the resin. The solution was shaken vigorously with chloroform (50 ml) and the chloroform layer (10 ml) was drawn off into a 25 ml graduated cylinder and shaken with boric acid buffer (5 ml), 0.1M, pH 10. The boric acid solution was pipetted into a quartz cuvette for

reading in a spectrophotofluorometer (Aminco-Bowman) at activation wavelength 315 nm, and fluorescence wavelength 410 nm (uncorrected). An internal standard curve was constructed using 10.0, 20.0, 30.0, and 40.0 µg/ml sodium salicylate in cat urine (s.g. 1.010).

G. Modified method for the determination of salicylate.

The above method was modified by omitting the procedure using the microresin column. This modification resulted in a nine fold increase in the amount of salicylate recovered. In this manner, larger amounts of salicylate were obtained for subsequent chromatography.

Instead of adsorbing the salicylate onto the IRA401 resin, proceed to the third extraction with 50 ml of chloroform and subsequent re-extraction into 0.1M borate buffer, (pH 10).

Although in the original method of Aidie (1967) only about 10% of a quantity of salicylate could be recovered, the recovery was directly proportional to the concentration of the original sample since a linear standard curve was obtained after extracting various salicylate standards and subsequent fluorometric assay.

The modified method of Aidie (1968) was useful when the purity of the salicylate was not important but when it was desirable to obtain large quantities of salicylate for assay involving paper chromatography.

However, when a fluorometric assay was required the original method of Aidie (1967) was followed since removal of substances interfering with the fluorometric assay was essential.

Method of Aidie (1967)

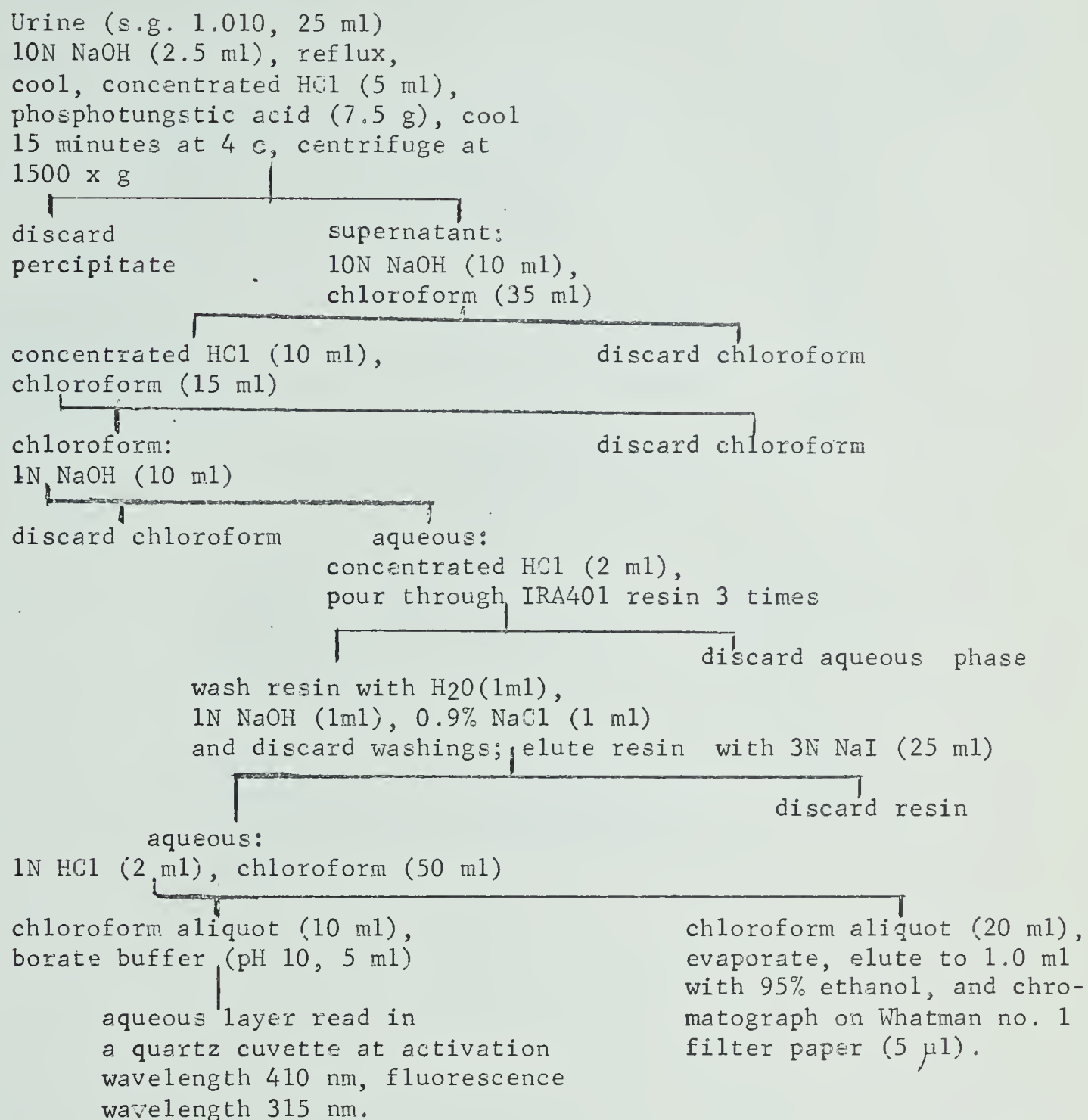
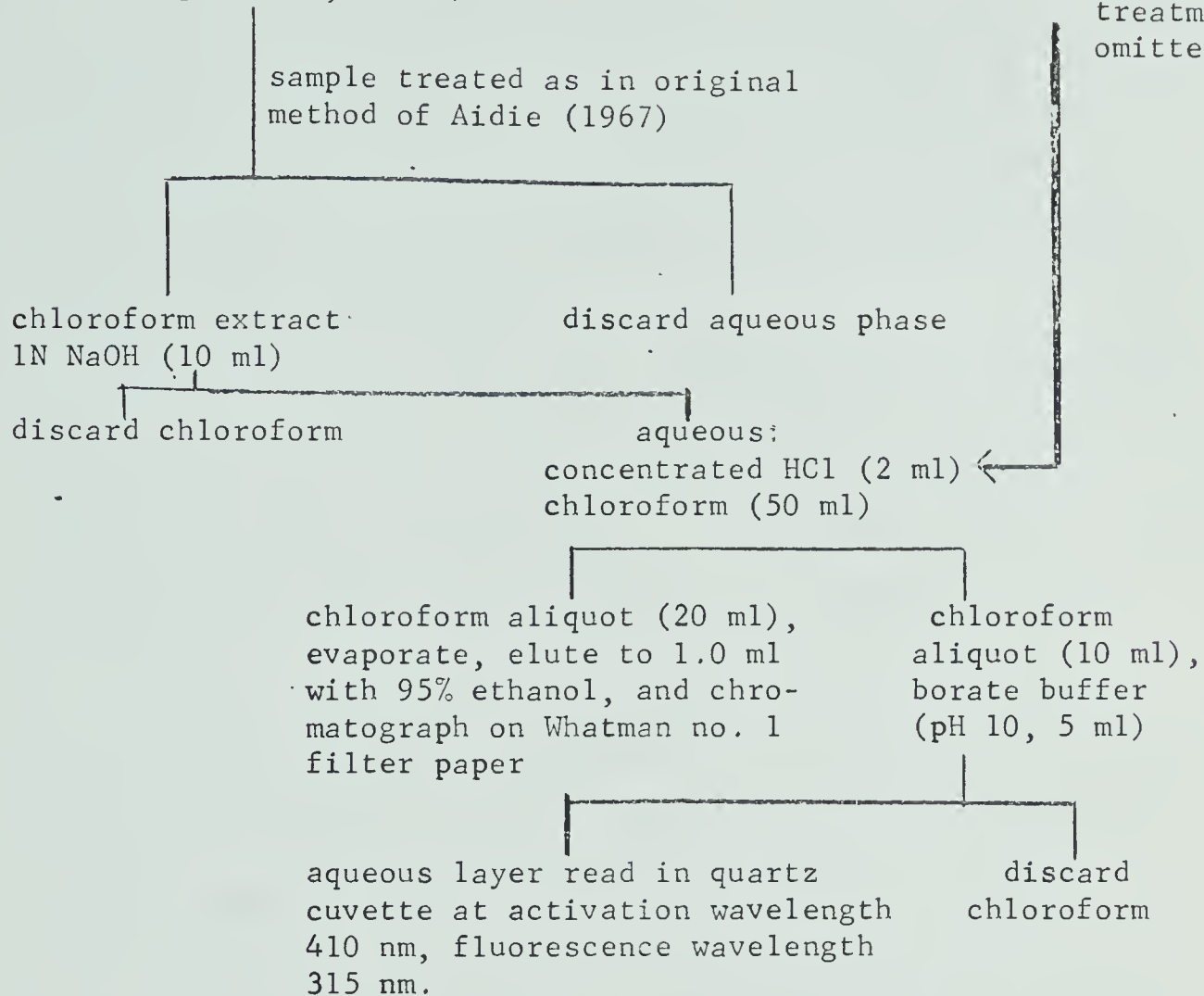


figure 3

Modified method of Aidie (1968)

Urine (s.g. 1.010, 25 ml)



Note:
IRA401 resin
treatment
omitted.

figure 4

H. Collection of cat urine.

Each cat was placed in a specially prepared metabolism cage to facilitate urine collection. The cage consisted of a wire mesh floor under which was placed an inclined metabolism pan. A short hose was used to connect the outlet from the metabolism pan to a bottle as shown in figure 2.

At the time of collection, the metabolism pan was washed with 25 ml of distilled water, and the water was allowed to drain into the collecting bottle. A few drops of toluene were added to the bottle before collection to retard bacterial growth.

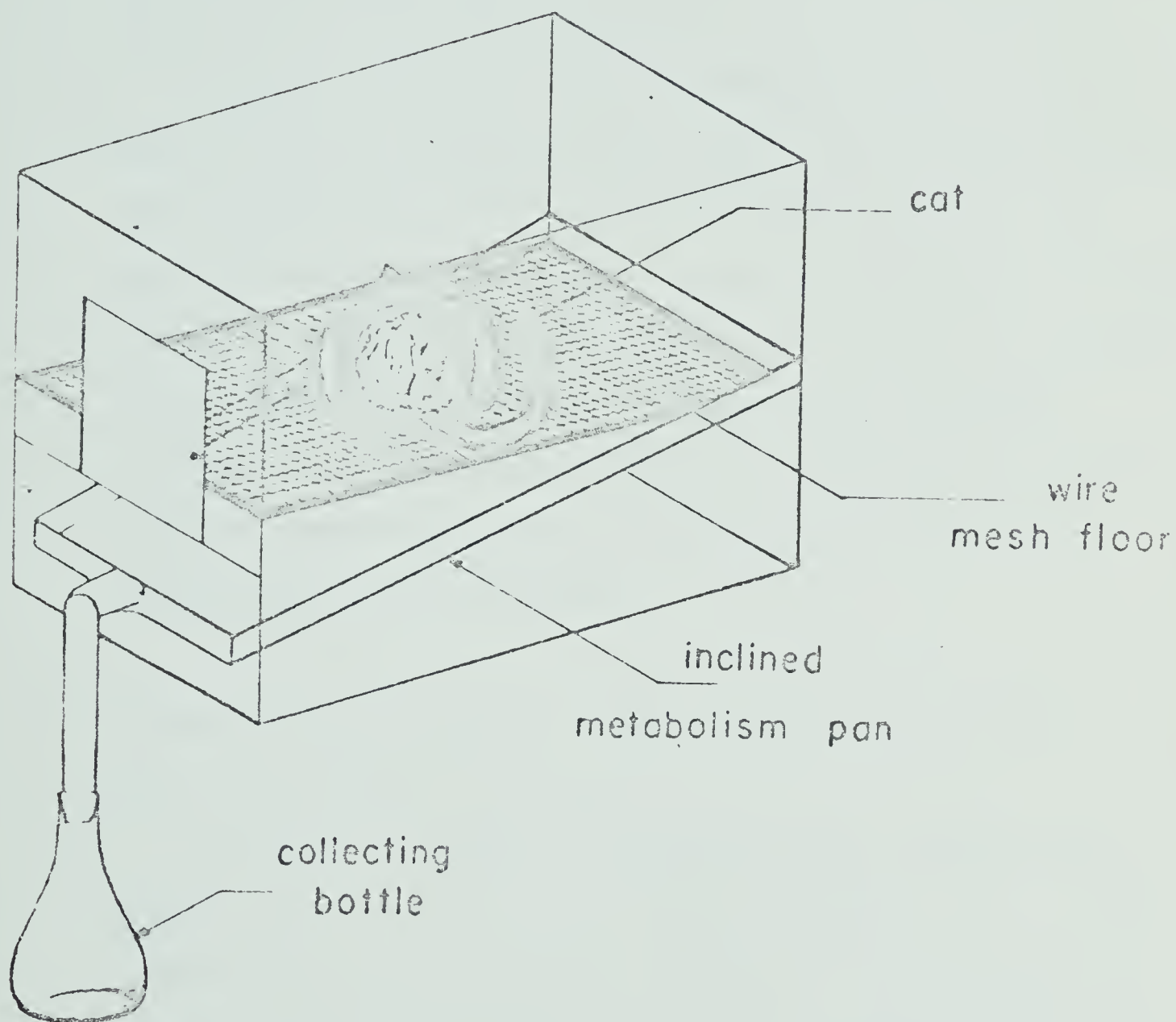
I. The attempted recovery of salicylate from cat urine following oral administration of sodium salicylate, or o-hydroxybenzyl alcohol, and intraperitoneal administration of o-cresol.

Three male cats (5.1 kg, 2.3 kg, and 4.0 kg) were each given an oral dose of sodium salicylate (50 mg/kg, 25 mg/kg and 10 mg/kg respectively) in gelatin capsules, and placed in metabolism cages. Urine excreted over a 24 hour period was assayed.

Three male cats (2.4 kg, 3.5 kg, and 3.3 kg) were given o-hydroxybenzyl alcohol (100 mg/kg, 50 mg/kg, and 25 mg/kg respectively) in gelatin capsules. The cats were placed in metabolism cages and the urine collected was removed after 24 hours and assayed.

figure 5

Cage for Urine Collection



Three male cats (4.7 kg, 2.5 kg, and 2.3 kg) were injected intraperitoneally (50 mg/kg, 25 mg/kg, and 10 mg/kg) with a 50%, 25%, and 10% solution respectively, of o-cresol in 95% ethanol. A control cat (3.1 kg) was injected with 95% ethanol (0.1 ml/kg) intraperitoneally. The cats were placed in metabolism cages and the urine was removed after 24 hours.

The urine was then assayed for salicylate using the method of Aidie (1967) and its modified form (1968). In the unmodified extraction procedure, the quantities of salicylate obtained were too small for subsequent chromatography to be feasible, therefore, the method was modified as described previously. The final chloroform extract (20 ml) was evaporated to dryness with nitrogen and redissolved in 1.0 ml of 95% ethanol. An aliquot of the solution (5 μ l) was then chromatographed on Whatman no. 1 filter paper using 20% w/v KCl as the solvent.

J. Efficiency of the recovery of TOCP, o-hydroxybenzyl alcohol, and o-cresol added to cat urine.

In order to evaluate the recovery of TOCP, o-hydroxybenzyl alcohol, and o-cresol, it was essential to know how much of each could be recovered when added to cat urine. This information is given in table 2, page 28 and was used in calculations of actual recoveries when these substances were administered parenterally.

K. Attempted recovery of o-hydroxybenzyl alcohol from cat urine following the administration of o-hydroxybenzyl alcohol and o-cresol.

The o-hydroxybenzyl alcohol level in the urine was assayed with the aid of a Microtech gas chromatograph as described previously. A standard curve was made using 0.5% o-hydroxybenzyl alcohol in chloroform. An experiment was performed to determine the efficiency of the procedure for extracting o-hydroxybenzyl alcohol from urine. A 0.5% solution of o-hydroxybenzyl alcohol in cat urine (25 ml) was extracted with two portions (25 ml) of chloroform. The pooled chloroform extracts were evaporated to dryness with nitrogen and redissolved in chloroform (25 ml). An aliquot of the solution (5 μ l) was chromatographed using a Microtech gas chromatograph.

To an aliquot (10 ml) of a 24 hour urine specimen was added concentrated HCl (5 ml) and water (35 ml). The solution was boiled 30 minutes in a water bath, cooled, and neutralized with 10N NaOH. The solution was extracted with two portions chloroform (25 ml). The pooled chloroform extracts were evaporated to dryness and eluted to 1.0 ml with 95% ethanol. An aliquot of the solution (5 μ l) was chromatographed using a Microtech gas chromatograph.

- L. Attempted recovery of o-cresol from cat urine following the intraperitoneal administration of o-cresol.

The hydrolyzed urine was assayed for o-cresol with the aid of a Microtech gas chromatograph as described previously. A standard curve for o-cresol was made using a 0.01% solution in 95% ethanol. An experiment was performed to determine the efficiency of the extraction of o-cresol from cat urine. 0.01% o-cresol, (100 ml) in cat urine was extracted with two portions (50 ml) of chloroform. The pooled chloroform extracts were evaporated to dryness with nitrogen and dissolved in 100 ml of 95% ethanol. The ethanol solution (5 μ l) was chromatographed.

To an aliquot (10 ml) of urine from a o-cresol treated cat were added concentrated HCl (5 ml) and water (35 ml). The solution was placed in a boiling water bath for 30 minutes, cooled, and neutralized with 10N NaOH. The final volume was adjusted to 100 ml with water:

- a) An aliquot of the solution (10 ml) was tested for phenols using Folin-Ciocolteau method (1927).
- b) A second aliquot of the solution (10 ml) was extracted with two portions (5 ml) of chloroform. The pooled chloroform extracts were evaporated to dryness with nitrogen and dissolved in 95% ethanol (1.0 ml). The ethanol solution was then subjected to gas chromatography and the concentration of o-cresol was calculated from the peak heights.

M. Tri-o-cresyl phosphate and its metabolites.

Three male cats weighing between 2 and 4.5 kg were used for this experiment. The cats were injected intraperitoneally with a 10% emulsion of TOCP-³H (100 mg/kg) in 10% Tween 80. The radioactivity of an aliquot of the emulsion was first assayed before its administration to the cats.

Urine from all cats was collected for 48 hours before the intraperitoneal administration of the TOCP-³H. Urine samples were then collected at 24 hour intervals for 8 consecutive days. Salicylate determination was carried out using the modified method of Aidie (1968). Scintillation fluid (5 ml) was added to 0.1 ml of urine and the radioactivity was assayed.

The TOCP level in the urine was also assayed with the aid of a Microtech gas chromatograph, as described previously. A standard curve was made using 0.01% TOCP in chloroform. An experiment was performed to determine the efficiency of extracting TOCP from cat urine. A 0.01% solution of TOCP in cat urine (25 ml) was extracted with two portions (50 ml) chloroform. The pooled chloroform extracts were evaporated to dryness with nitrogen and redissolved in chloroform (25 ml). An aliquot of the solution (5 μ l) was subjected to gas chromatography.

Urine (10 ml) was extracted with two portions (20 ml) chloroform. The pooled chloroform extracts were evaporated to dryness with nitrogen and redissolved in chloroform (1.0 ml). The solution (5 μ l) was then gas chromatographed.

RESULTS

1. Assay of enzyme activity

a. acid phosphatase

The potato acid phosphatase possessed its optimum activity at a pH of approximately 5.4 using disodium phenyl phosphate (48 mM) as substrate. These results are expressed in figure 6. The acid phosphatase failed to hydrolyze TOCP at a pH of 5.4.

b. alkaline phosphatase

The calf mucosal alkaline phosphatase possessed its optimum activity at a pH of approximately 9.0 using disodium phenyl phosphate (48 mM) as substrate. The alkaline phosphatase failed to hydrolyze TOCP at a pH of 9.

c. phosphodiesterase

The brussel sprout phosphodiesterase was active at a pH of 6.6 using sodium diphenyl phosphate (200 mM), but failed to hydrolyze TOCP or TPP at this pH.

Failure of hydrolysis of TOCP was indicated by the fact that no phenol formation was observed.

figure 6

pH-Activity Curve for
Potato Acid type II Phosphatase

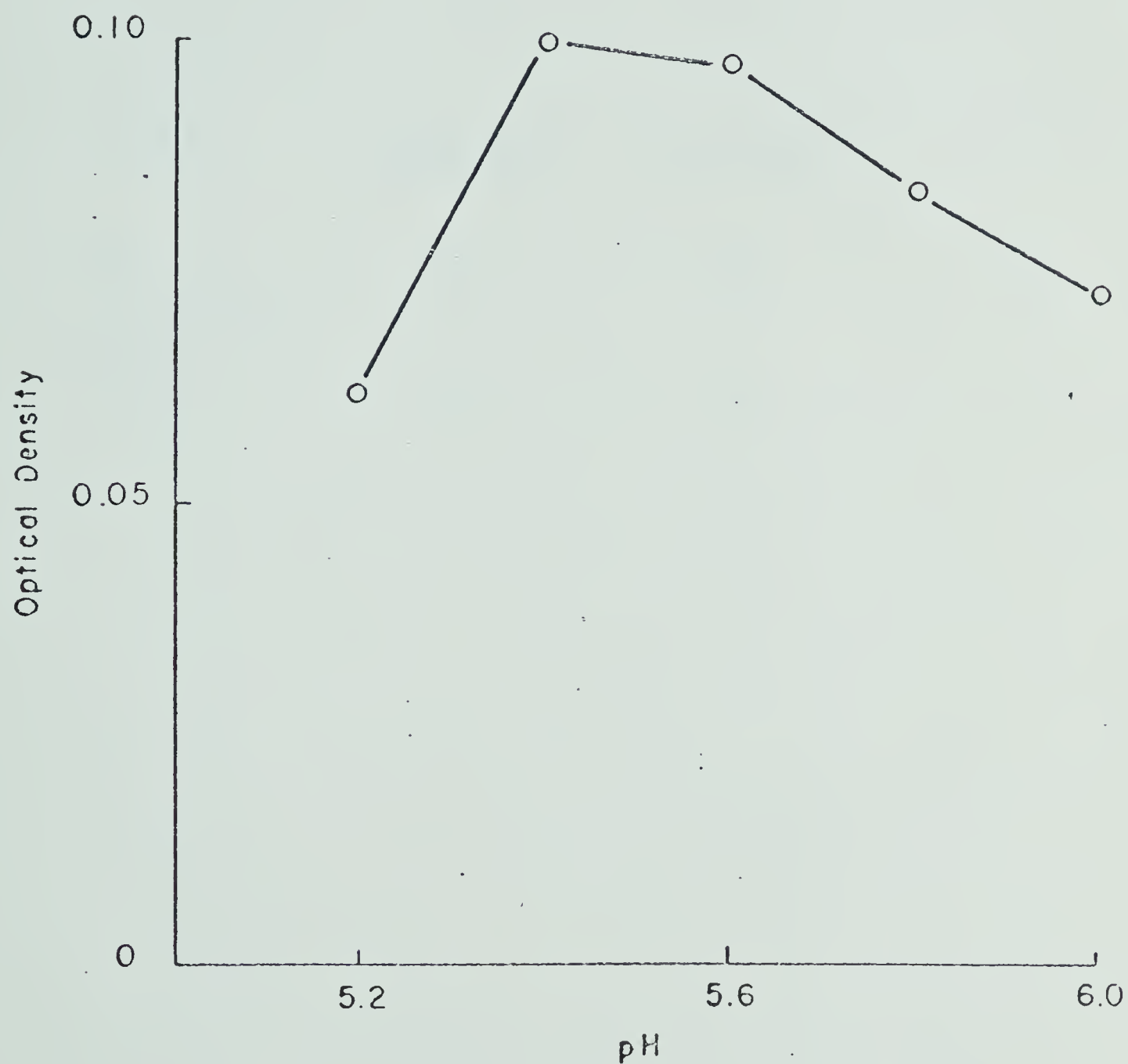


Table 2

Summary of the efficiency of the recovery of
TOCP, o-hydroxybenzyl alcohol, and o-cresol
added to cat urine.

compound	amount added (μ moles/ml)	amount recovered (μ moles/ml)	% efficiency
TOCP	0.28	0.17	60
o-Hydroxybenzyl alcohol	4.0	2.2	55
o-Cresol	0.90	0.36	40

2. Recovery of salicylate from cat urine after its oral administration.

The standard curve obtained using the fluorometric salicylate assay (Aidie, 1967) is shown in figure 7. Sodium salicylate (0.92 mmol, 0.28 mmol, and 0.12 mmol) was recovered from the urine of three cats 24 hours after the administration of sodium salicylate (1.60 mmol, 0.42 mmol, and 0.25 mmol respectively) and the average recovery represented approximately 60% of the administered oral dose.

3. Recovery of salicylate in cat urine after oral administration of o-hydroxybenzyl alcohol.

In a control experiment o-hydroxybenzyl alcohol was added to cat urine (s.g. 1.010) so that the concentration was 19.3 mM. This was done to determine whether o-hydroxybenzyl alcohol gave false positive results with the assay for salicylate. No apparent salicylate was found when the urine was assayed for salicylate using the method of Aidie (1967).

After the oral administration of o-hydroxybenzyl alcohol to three cats (1.64 mmol, 1.41 mmol, and 0.67 mmol), salicylate excretion was determined by the method of Aidie (1967) and an increase in urine salicylate content of approximately 0.48 mmol, 0.56 mmol, and 0.33 mmol respectively was obtained.

These cats excreted between 0.2 μ mol to 0.4 μ mol/day of urinary salicylate during a control period prior to the administration

of sodium salicylate. This increase in urinary salicylate accounted for about 40% of the administered o-hydroxybenzyl alcohol.

4. Recovery of o-hydroxybenzyl alcohol from cat urine after the oral administration of o-hydroxybenzyl alcohol and intraperitoneal administration of o-cresol.

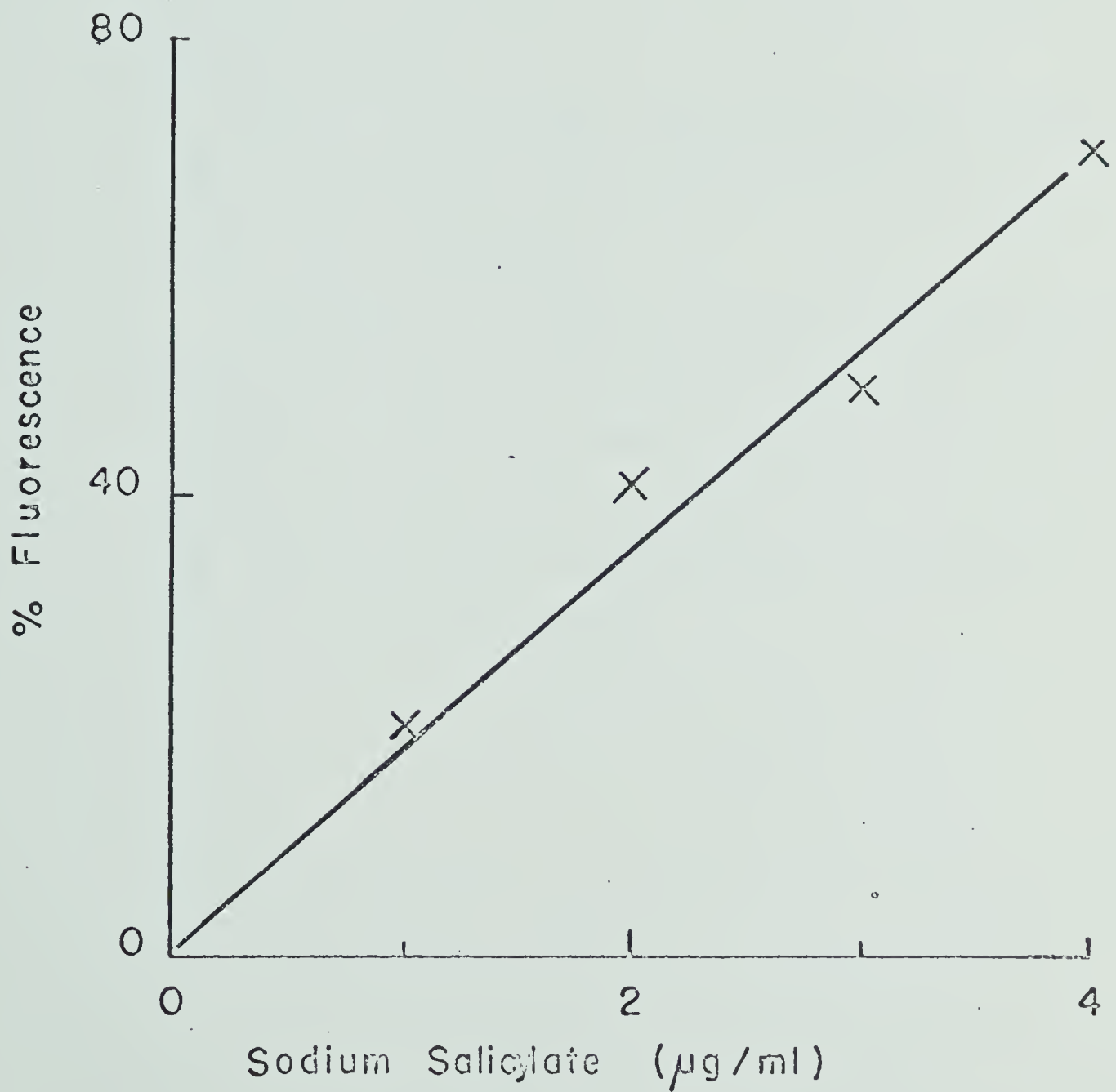
Figure 8 shows a linear relationship between o-hydroxybenzyl alcohol concentration and peak height obtained with the aid of a Microtech gas chromatograph.

Approximately 0.079 mmole and 0.13 mmole of o-hydroxybenzyl alcohol were excreted in the urine 24 hours after the oral administration of 0.67 mmole and 1.41 mmole respectively. This recovery represented approximately 10% of the administered dose.

No o-hydroxybenzyl alcohol was found in cat urine after the intraperitoneal administration of o-cresol to cats.

Figure 7

Standard Curve for
Salicylate Assay
(Aidie, 1967)



5. Recovery of o-cresol and salicylate from cat urine following the intraperitoneal administration of o-cresol.

a. Test for phenols.

The standard curve for o-cresol using the Folin-Ciocolteau method (1927) is shown in figure 9. The results in figure 10 show that a significant increase in phenols can be demonstrated in cat urine 24 hours after the intraperitoneal administration of o-cresol.

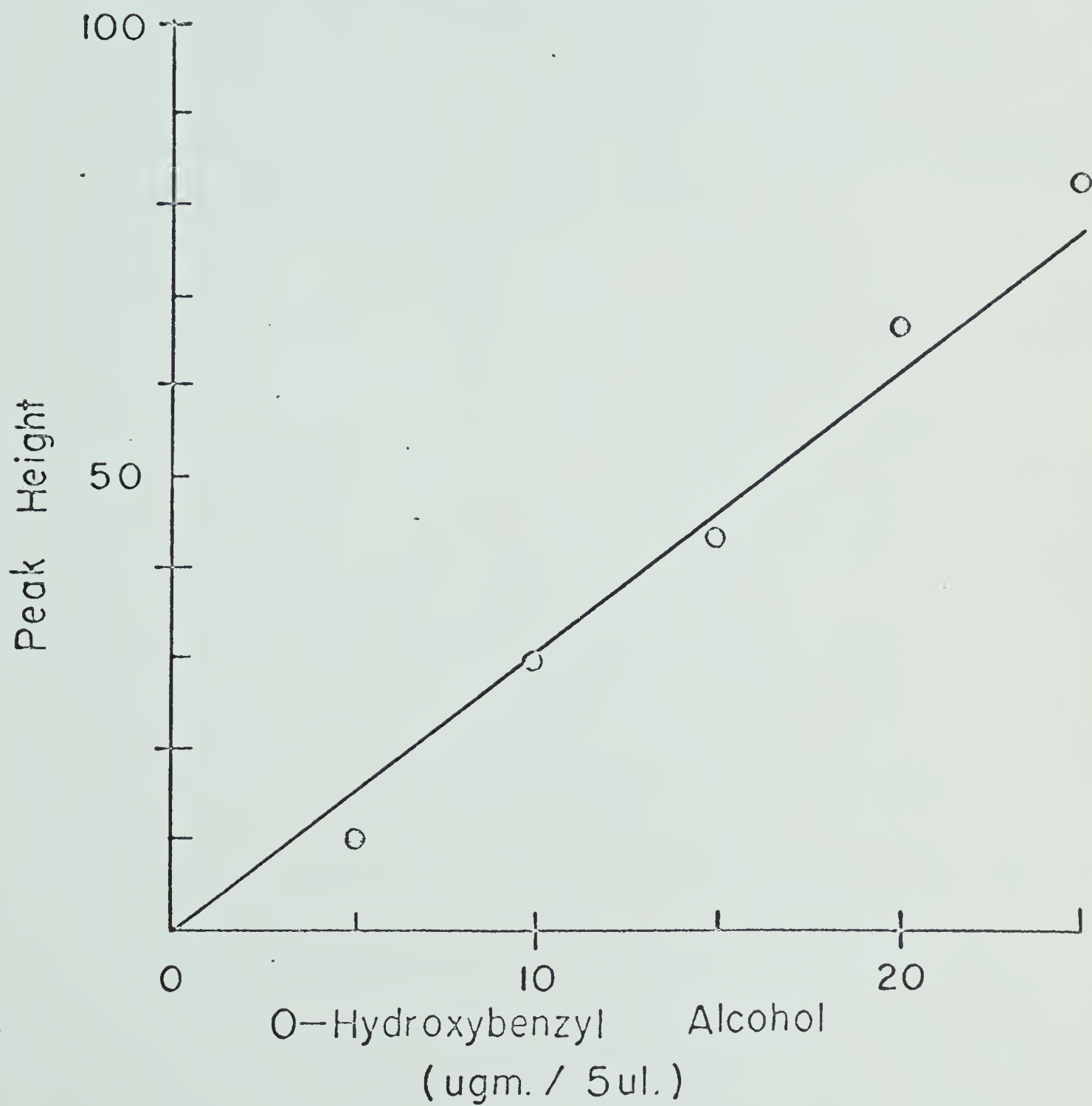
b. Assay for o-cresol.

Figure 11 shows a linear relationship between o-cresol concentration and peak height obtained with the aid of a Microtech gas chromatograph.

Approximately 0.79 mmole, 0.38 mmole, and 0.082 mmole of o-cresol were excreted in the urine 24 hours after intraperitoneal injection of 2.18 mmole, 0.58 mmole, and 0.20 mmole respectively of o-cresol. This recovery averaged 47% of the administered dose. Only a trace of o-cresol was found in the urine 48 hours after its administration.

figure 8

Standard Curve for
o-Hydroxybenzyl alcohol
using a Microtech Gas Chromatograph



c. Assay for salicylate.

A linear standard curve was obtained using the modified method of Aidie (1968) as shown in figure 12. In this method a greater amount of salicylate was extracted, and therefore this latter method proved to be more useful for subsequent paper chromatography.

Approximately 0.05 mmole, 0.023 mmole, and 0.009 mmole of sodium salicylate were excreted in the urine after the intraperitoneal administration to cats of 2.18 mmole, 0.58 mmole, and 0.20 mmole, respectively of o-cresol.

figure 9

Standard Curve for
o-Cresol in Cat Urine
(Folin - Ciocolteu)

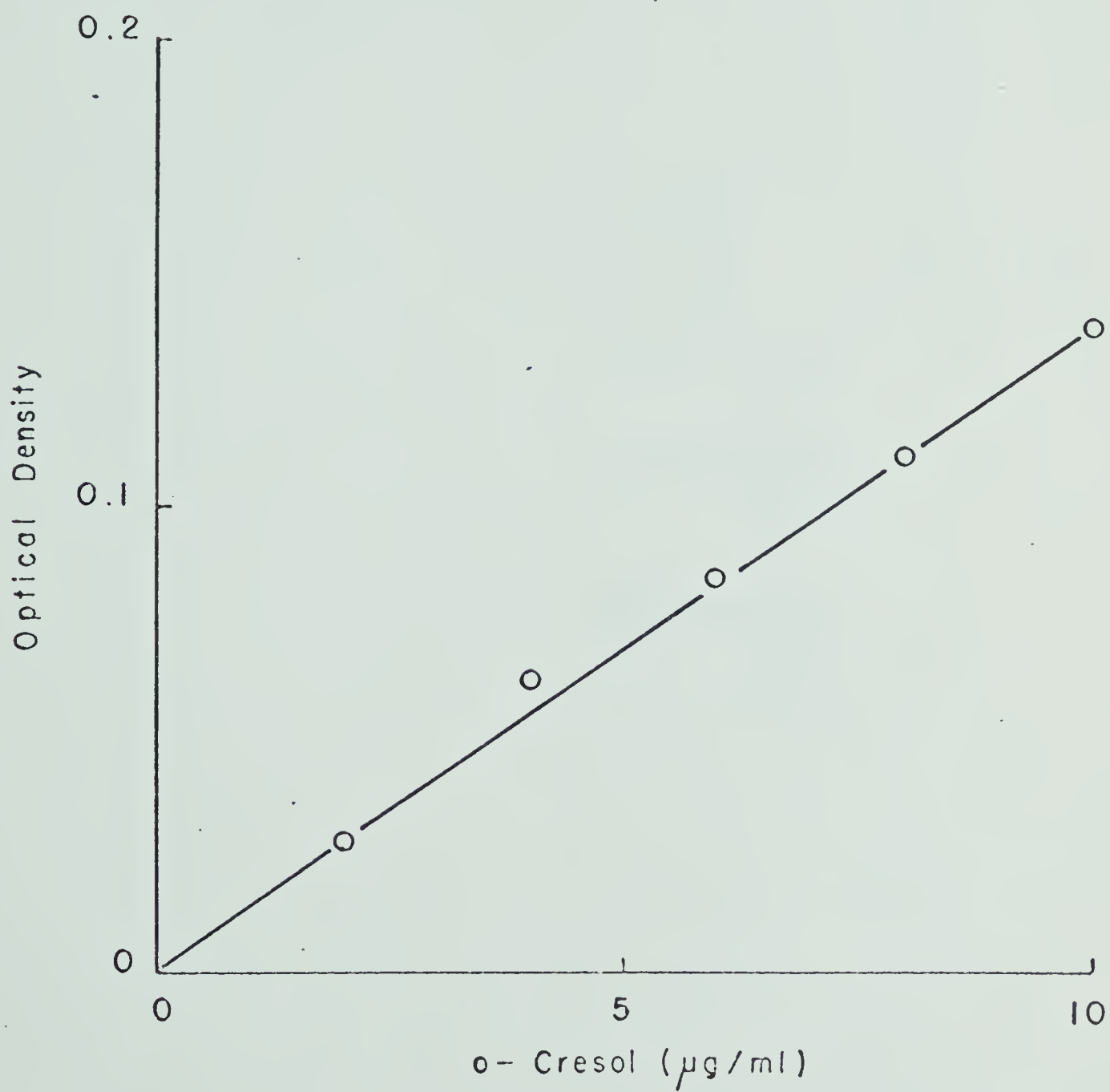


figure 10

Amount of phenol excreted in cat urine
after administration of O - Cresol
to cats.

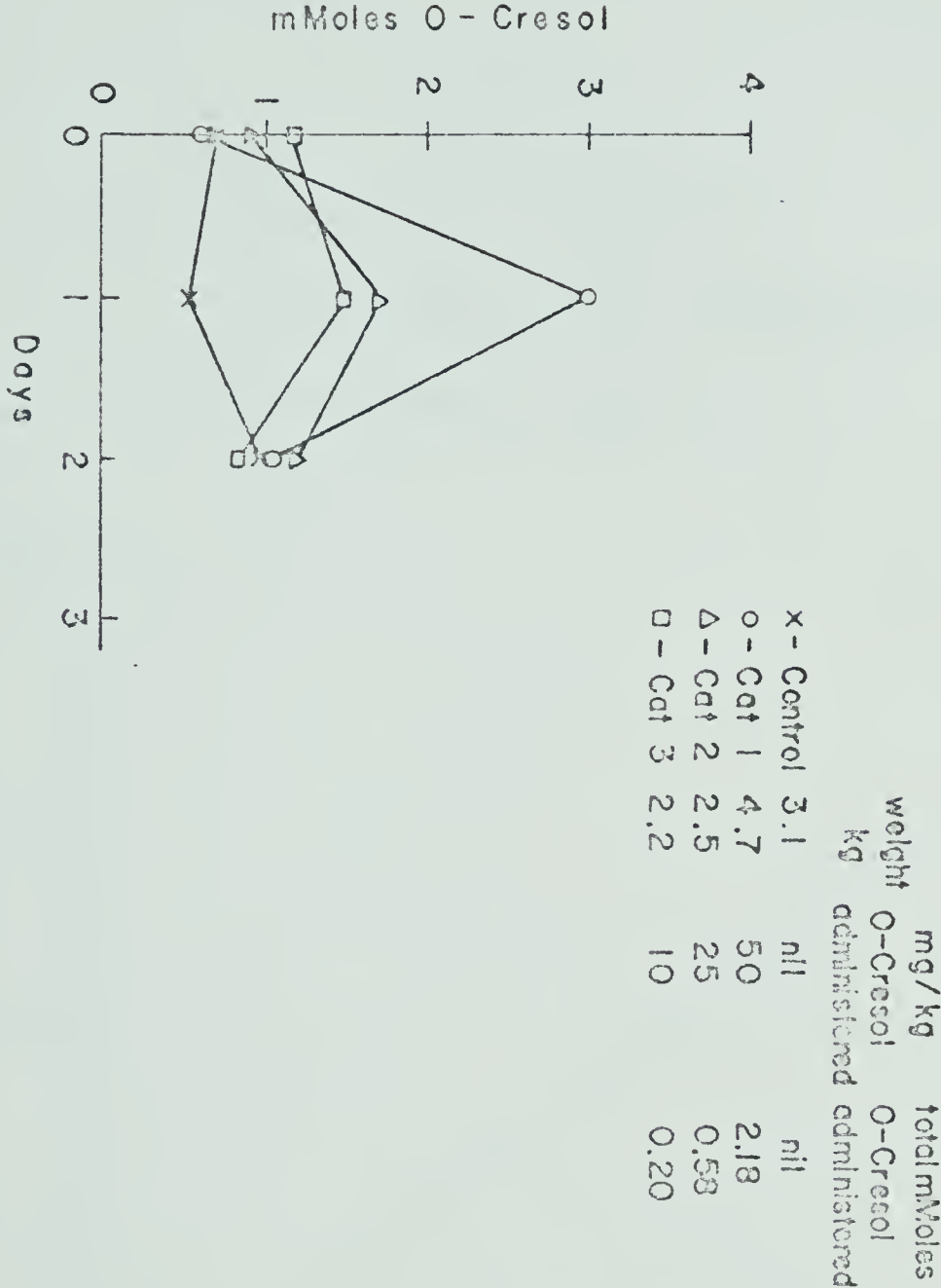
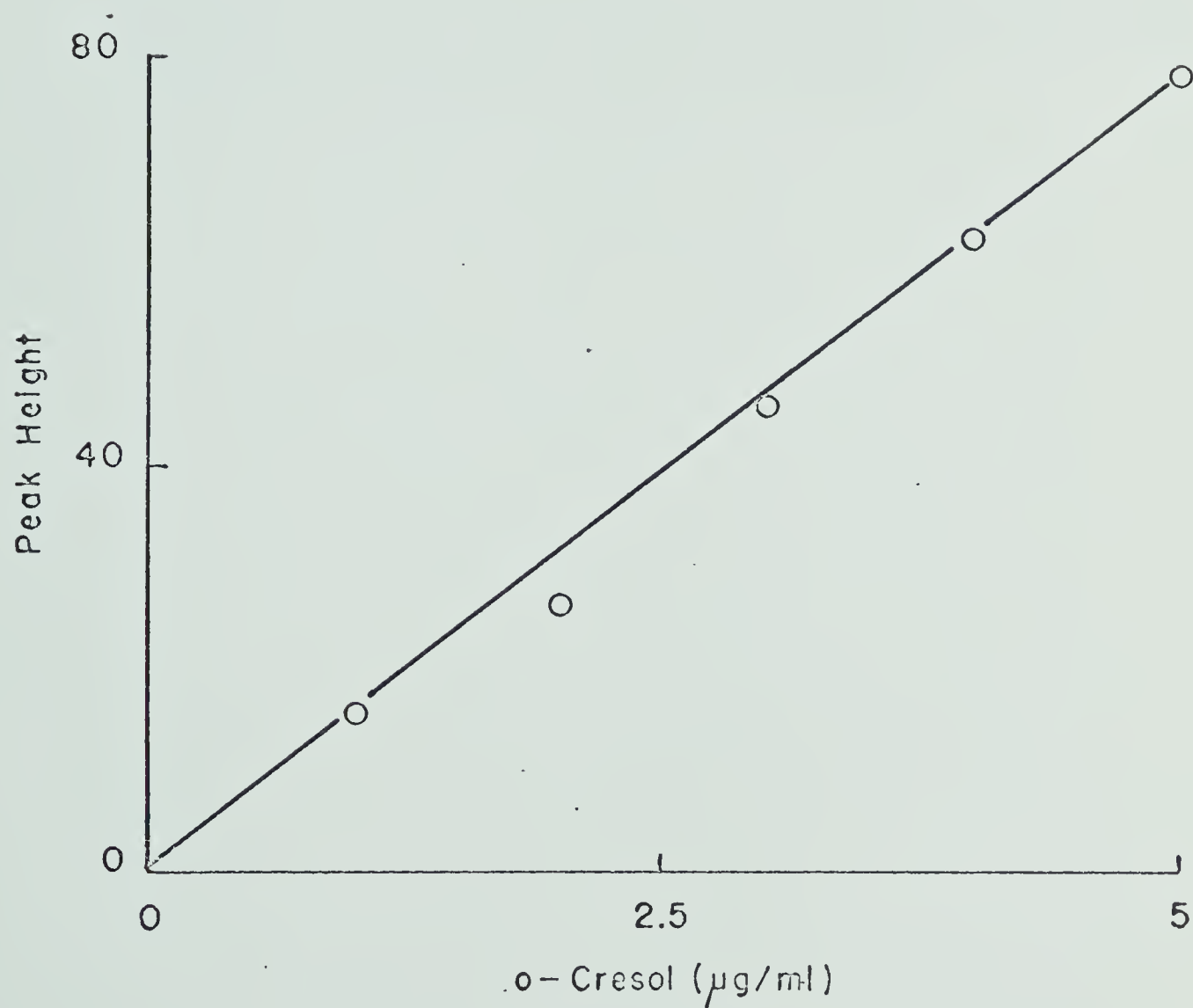


figure 11

Standard Curve for
o-Cresol using a Microtech
Gas Chromatograph



6. Column chromatography.

The results from a chromatographic purification of TOCP-³H are expressed in figure 13. The optical density and radioactivity are plotted against the fraction number. Fraction 4 to 8 which had a high optical density together with a corresponding increase in radioactivity were pooled and reserved for a subsequent experiment. The presence of TOCP-³H was confirmed with the aid of an ultraviolet absorption spectrum. The remaining fractions were discarded since the results suggested that these fractions were contaminated with material other than TOCP-³H.

Standard Curve for Salicylate Assay
using the Modified Method of Aidie (1968)

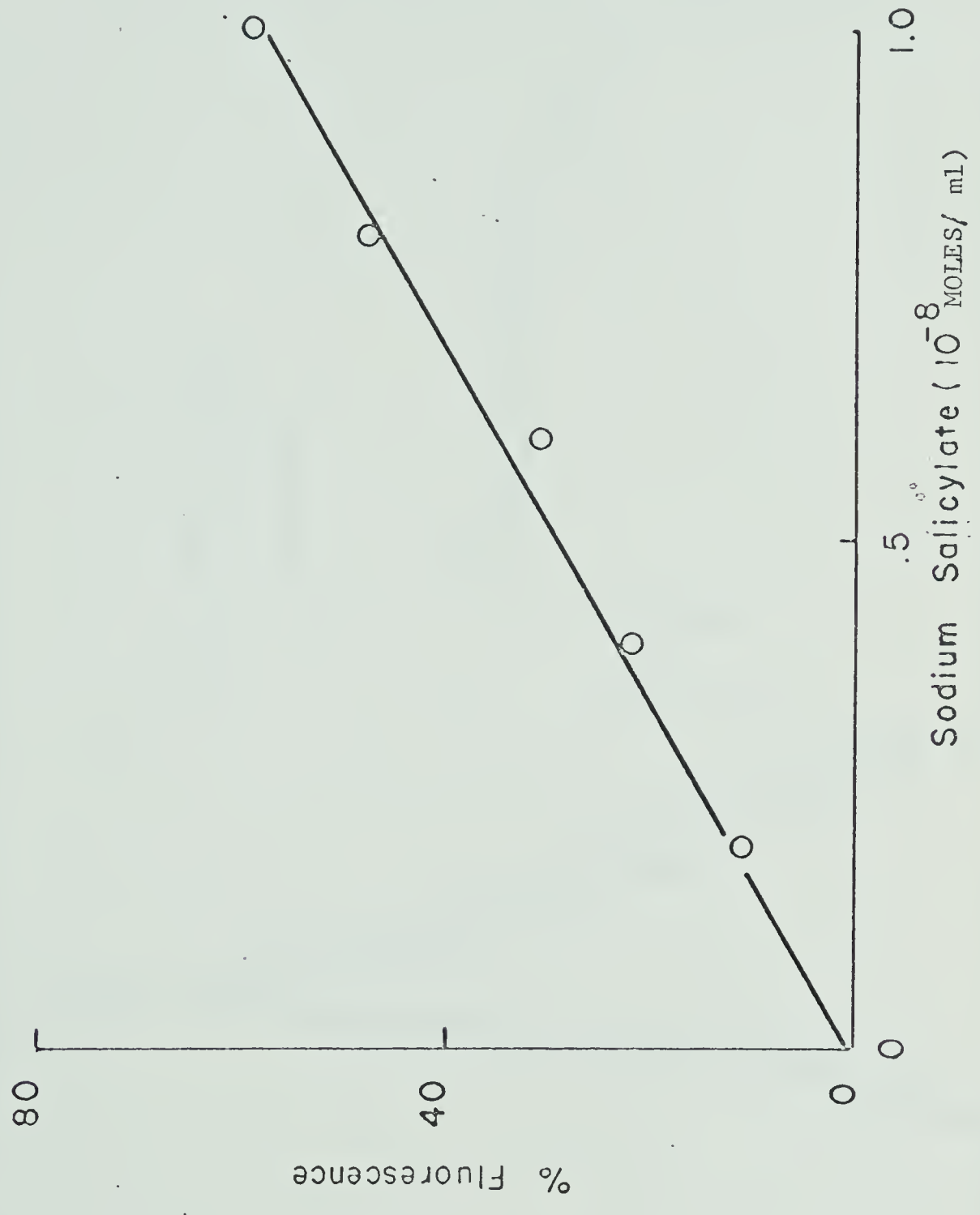
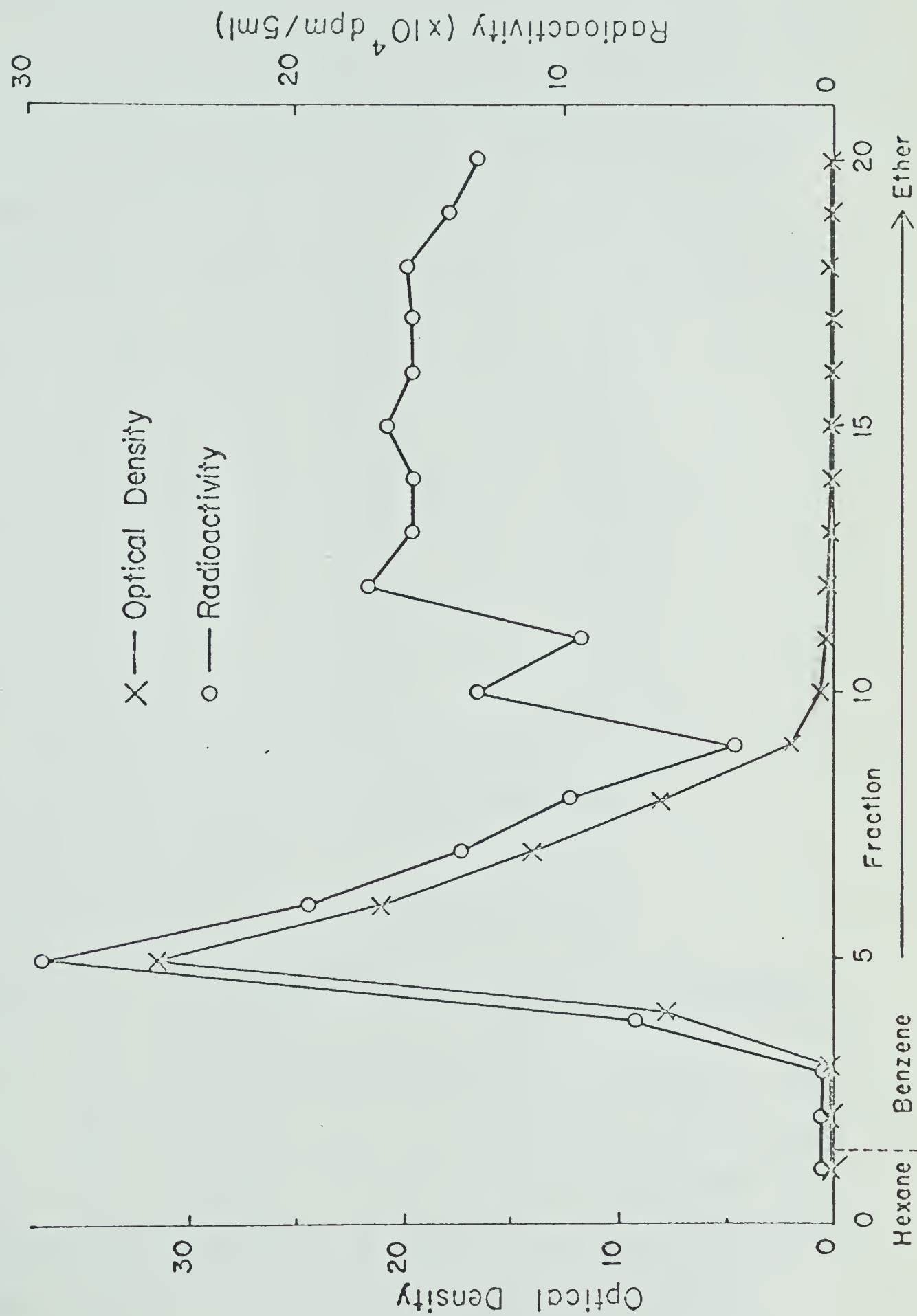


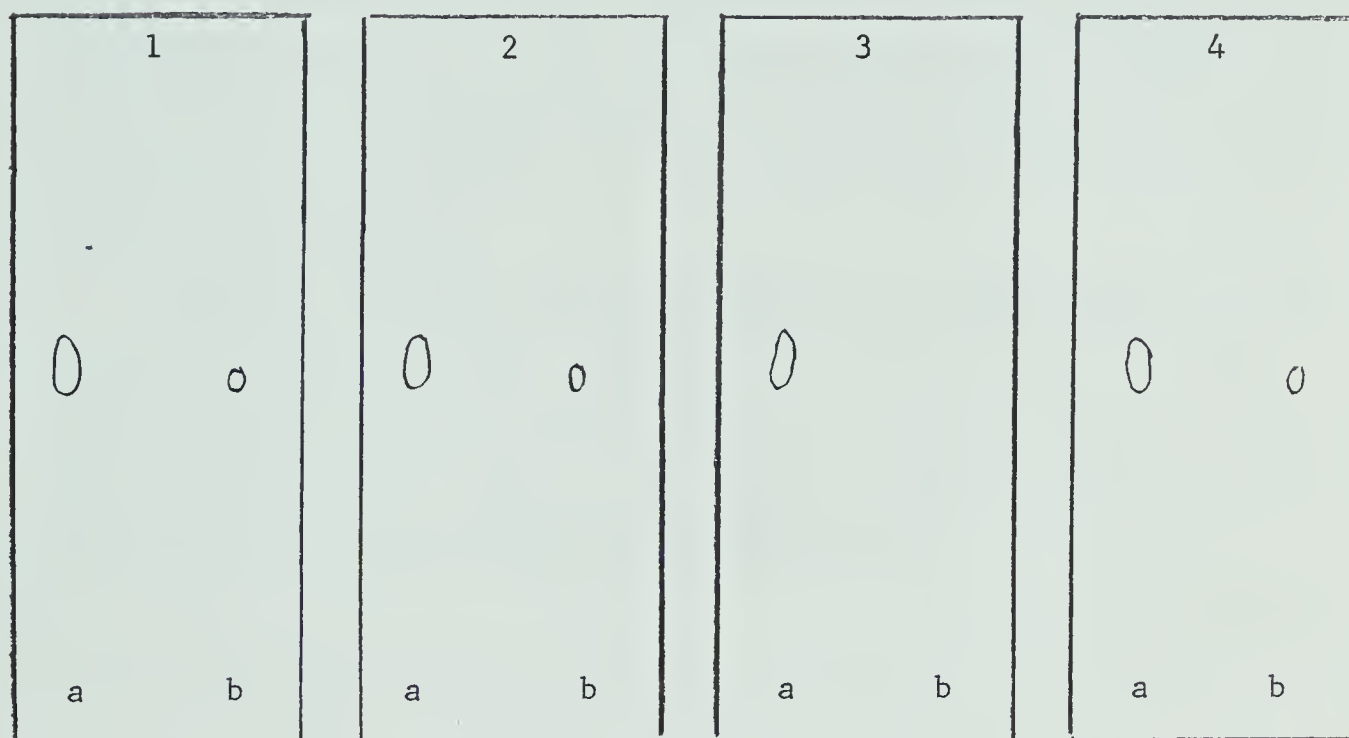
figure 13

Adsorption Column Chromatography of TOCP- ^3H



7. Paper chromatography.

The following are examples of paper chromatographs of salicylic acid obtained from urine extracts (Aidie, 1967) after the administration of sodium salicylate, o-hydroxybenzyl alcohol, and o-cresol to cats:



(a) 5 μ l 0.1% salicylic acid in 95% ethanol.

(b) 5 μ l extract

The results are summarized in the following table:

Paper no.	Material administered to cat	R _f		procedure
		(a)	(b)	
1	salicylate	0.66	0.66	(Aidie, 1967)
2	o-hydroxybenzyl alcohol	0.66	0.66	(Aidie, 1967)
3	o-cresol	0.66	*	(Aidie, 1967)
4	o-cresol	0.66	0.66	(Aidie, 1968)

* This chromatogram demonstrates the value of the modified procedure of Aidie since insufficient salicylate is available for subsequent chromatography after elution from IRA401 resin.

8. Tri-o-cresyl phosphate and metabolites.

No significant amount of salicylate was recovered from the urine of the three cats during 8 consecutive days after the intraperitoneal administration of TOCP-³H (100 mg/kg).

However, a significant amount of radioactivity was recovered from the urine. The radioactivity recovered from urine during 8 consecutive days of assay as shown in figure 14 and table 4 represented approximately 17%, 7%, and 13% respectively of the TOCP-³H administered.

Figure 15 shows a linear relationship between TOCP concentration and peak height obtained with the aid of a Microtech gas chromatograph. TOCP-³H was recovered from cat urine after the administration of TOCP-³H to three cats.

table 3

Assay of cat urine for TOCP and tritium following the administration of TOCP-³H to cats.

cat	days*	Gas chromatographic assay of TOCP in urine (% administered dose)	radioactivity in urine (% administered dose)
1	0	0	0
	2	3.6	4.5
2	0	0	0
	6	2.0	1.9
3	0	0	0
	7	2.2	2.2

* This corresponded to the day on which the greatest excretion of radioactivity occurred.

figure 14

Radioactivity Assayed in Cat Urine
After the Administration of TOCP- H^3

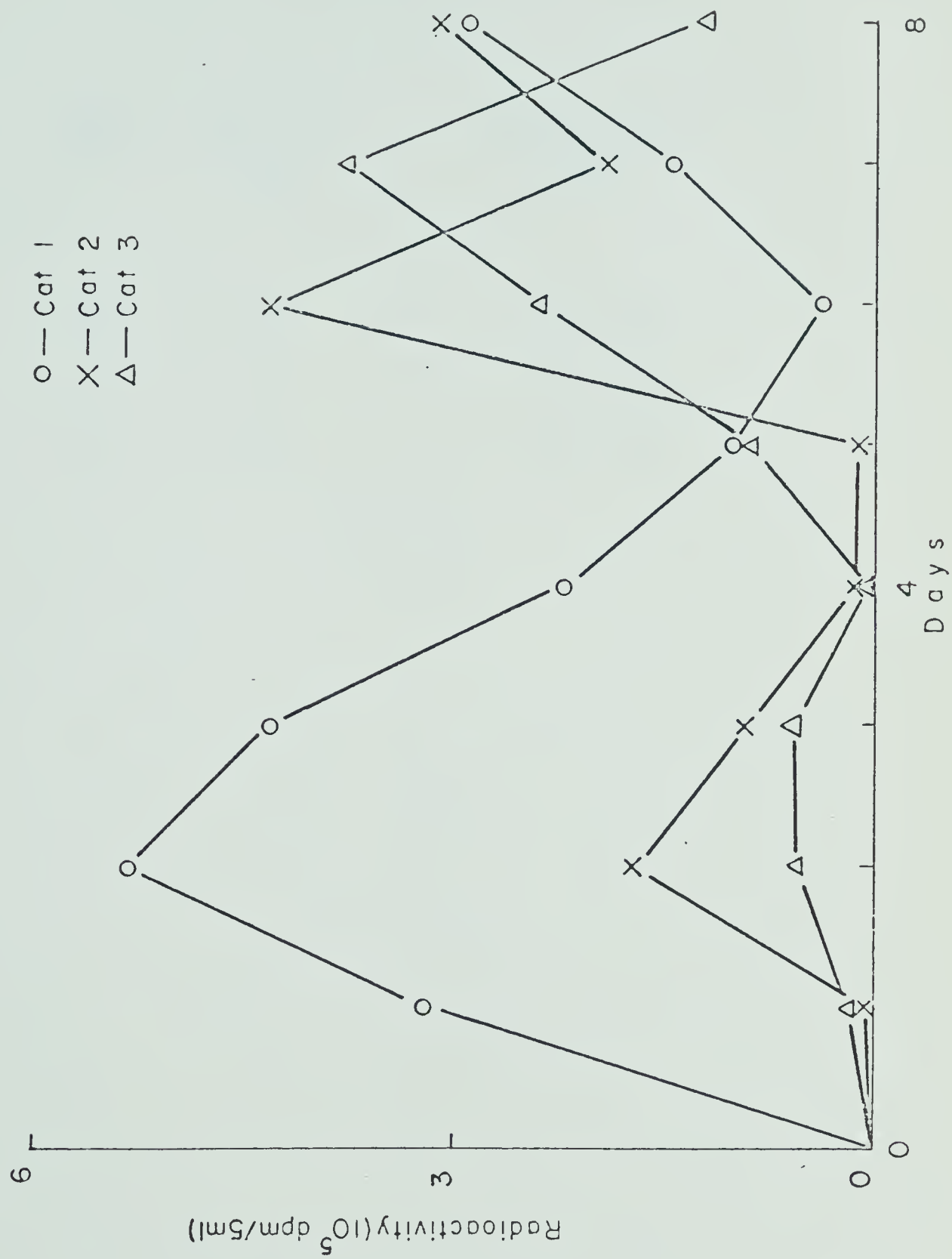


table 4

Amount of tritium administered to cats.

cat	weight (kg)	TOCP- ³ H administered (mg)	Radioactivity administered (dpm)
1	3.0	300	1.19×10^7
2	3.3	330	1.28×10^7
3	2.2	220	8.71×10^6

figure 15

Standard Curve for
TOCP using a Microtech Gas
Chromatograph

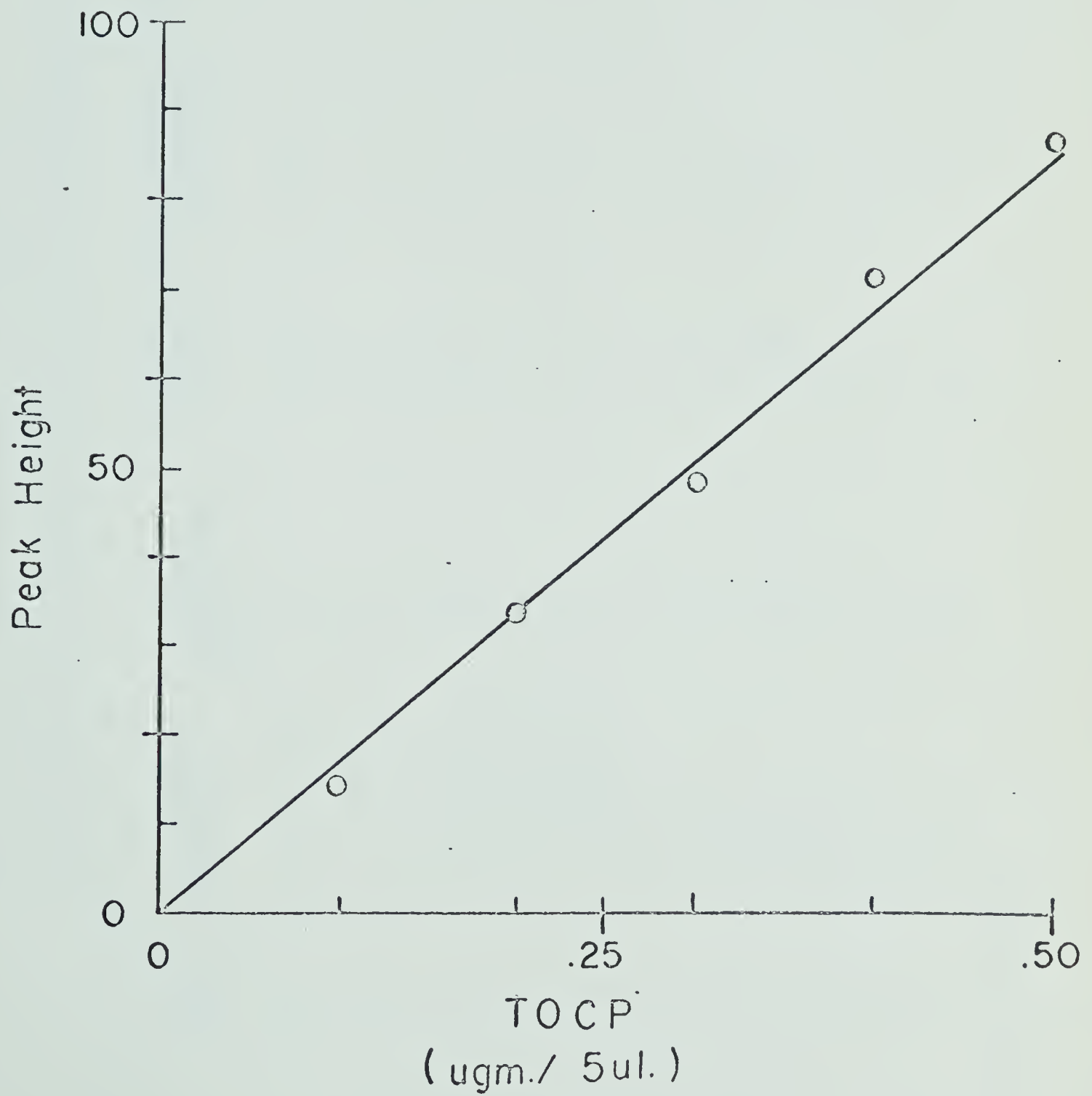


table 5

Summary of the results obtained for the metabolism and excretion of TOCP, salicylate, o-cresol, and o-hydroxybenzyl alcohol.

cat	weight (kg)	material administered	amount administered (mmole)	Material recovered from urine (mmole)/24 hours			
				sodium salicylate	o-hydroxy- benzyl alcohol	o-cresol	TOCP
1	5.1	Na salicylate :	1.60	0.92	NA**	NA	NA
2	2.3	Na salicylate	0.41	0.28	NA	NA	NA
3	4.0	Na salicylate	0.25	0.12	NA	NA	NA
4	2.4	o-Hydroxybenzyl alcohol	1.64	0.48	NA	NA	NA
5	3.5	o-Hydroxybenzyl alcohol	1.41	0.56	0.13	NA	NA
6	3.3	o-Hydroxybenzyl alcohol	0.67	0.33	0.08	NA	NA
7	4.7	o-Cresol	2.18	0.051	NA	0.79	NA
8	2.5	o-Cresol	0.58	0.023	nil	0.38	NA
9	2.3	o-Cresol	0.20	0.009	nil	0.08	NA
10	3.0	TOCP- ³ H	0.83	nil	NA	NA	0.03
11	3.3	TOCP- ³ H	0.91	nil	NA	NA	0.02
12	2.2	TOCP- ³ H	0.60	nil	NA	NA	0.01

** NA=not assayed

Discussion

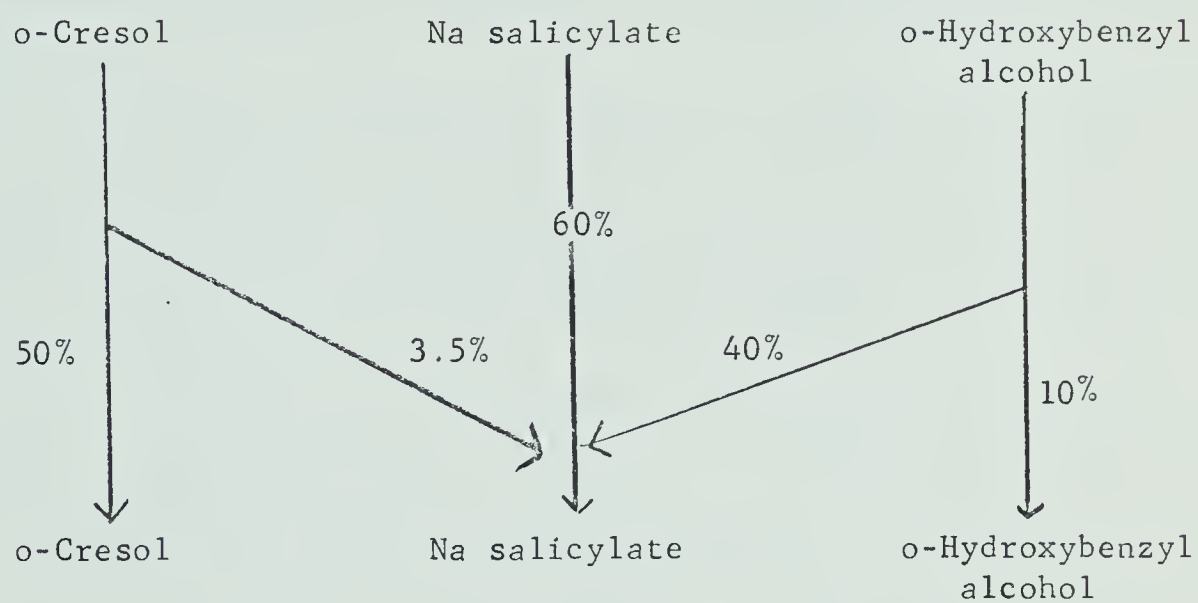
It has been demonstrated that TOCP is resistant to hydrolysis by an acid phosphatase, an alkaline phosphatase, and a phosphodiesterase. We felt that hydrolysis of TOCP by phosphatase may not occur in vivo until after TOCP has first been hydroxylated. By considering the metabolite, CBDP we subsequently suggested that should an hydroxylation reaction occur on any of three methyl groups of TOCP the resulting alcohol might then be susceptible to further metabolic changes.

Before this hypothesis could be tested, it was shown by Eto & Casida (1967) that an hydroxylation reaction does indeed occur prior to other molecular changes, in the course of the conversion of TOCP to CBDP.

Our evidence lends support to that of Eto & Casida (1967) who have shown the resistance of triphenyl phosphate to hydrolysis by various phosphatases such as those found in crude snake venom, porcine, bovine, and human plasma albumin fractions.

These workers found that porcine plasma, bovine plasma, human plasma as well as crude snake venom do phosphorolyse and cyclize di-o-tolyl o-(α -hydroxy) tolyl phosphate thereby producing the activated metabolite, CBDP. In addition, they found that the activity involved in this cyclization reaction is not due to enzymes that hydrolyze substrates such as p-nitrophenyl acetate or diphenyl phosphate, because the cyclizing enzyme is more stable to heat than are the enzymes hydrolyzing the other substrates.

In view of the extensive use of cats in pharmacology it was surprising to find that the metabolism and excretion of salicylate, o-hydroxybenzyl alcohol, and o-cresol in this species had not been reported. Our investigations involving the metabolism of these compounds in cats are summarized below:



These results indicate that a significant amount of salicylate can be recovered from cat urine after the administration to cats of salicylate itself or o-hydroxybenzyl alcohol.

Although salicylic acid is a possible metabolite of o-cresol, previous investigators did not find this metabolite excreted in the urine of other species after the administration of o-cresol. In this work a significant amount of salicylate (2-4%) was assayed in cat urine following the intraperitoneal administration of o-cresol to cats.

The lack of glucuronide formation in cats (Williams, 1959) is an interesting metabolic anomaly which seems to be unique for this species. Although cats can form conjugated sulfates, the inability for cats to form glucuronide conjugates might delay the excretion of phenolic compounds in urine and bile. This anomaly in cats might then explain the fact that the expected recovery of the various metabolites in urine was lower than expected. Furthermore, the lack of glucuronide conjugation suggests that cats, which are known to be extremely sensitive to phenols (Secord, 1967) might have an increased sensitivity to these compounds due to an inability to detoxify and excrete these materials quickly.

Since TOCP may be metabolized to yield o-hydroxybenzyl alcohol and/or o-cresol, then the presence of sodium salicylate in cat urine could provide evidence for the existence of these metabolic intermediates.

Eto & Casida (1962) have suggested that the requirements for the formation of o-hydroxybenzyl alcohol are the formation of CBDP and the subsequent reaction of this with "activated serine" to liberate the alcohol.

Thus, our failure to show salicylate in the urine of cats following TOCP administration suggest the following possibilities:

1. CBDP is not formed in the cat.
2. o-Hydroxybenzyl alcohol is not liberated after the formation of CBDP.
3. The formation of CBDP from TOCP may occur too slowly, or may be too limited, thereby restricting the production of sufficient o-hydroxybenzyl alcohol for subsequent metabolism and assay as salicylate.

The first possibility seems unlikely because the available evidence indicates that CBDP can be isolated from cat intestine after the administration of TOCP (Buttar, 1967). The second possibility also seems unlikely because the work of Eto & Casida (1962) strongly suggests the formation of o-hydroxybenzyl alcohol when CBDP reacts with an active serine.

The last possibility seems more acceptable since in this work it was observed that small amounts of tritium were detected in urine for each of 8 consecutive days after the administration of TOCP-³H to cats. These observations indicate that most of the administered

tritium is retained by the cat for a considerable time indicating that the metabolic processes that would facilitate excretion of TOCP are greatly reduced in this species.

The possibility should not be overlooked that substantial amounts of CBDP might be metabolized by pathways that do not yield o-hydroxybenzyl alcohol. Therefore, only a small quantity of the CBDP may react with the "active serine" of various enzymes such as acetylcholinesterase, thereby limiting the liberation of o-hydroxybenzyl alcohol in sufficient quantity for assay.

The appearance of more than one inhibitor of cholinesterase in the urine of rabbits and rats after the administration of TOCP to these animals, have been reported by Meyers et al. (1955). Although, in our investigations with cats, we did not assay the urine for CBDP or o-cresol, TOCP-³H was recovered from the urine after its administration to cats, and the quantities of TOCP represented 80-100% of the tritium present in the urine.

Some of the questions which remain unanswered are: Do cats excrete acetylcholinesterase inhibitors in their urine following TOCP administration in a manner similar to rodents (Meyers et al., 1955)?

Can the neurotoxicity of TOCP in some species such as the cat be related to its rate of hydroxylation?

Can CBDP be metabolized more quickly by rodents than by cats?

In view of the lack of information on the metabolism of o-hydroxybenzyl alcohol, o-cresol and salicylic acid in cats and particularly because of their failure to form the usual glucuronide conjugates, more complete studies on sulfate conjugation, gentisic acid formation, etc. should be carried out with this species.

Finally, a urinary salicylate assay should be performed following the administration of CBDP to cats. Such an experiment might add to our knowledge of CBDP metabolism.

Conclusions

It would appear that TOCP is resistant to hydrolysis by alkaline phosphatase, acid phosphatase, or phosphodiesterase, and may have to undergo hydroxylation before hydrolysis is possible.

o-Hydroxybenzyl alcohol and o-cresol can both be converted to salicylate and excreted in the urine by the cat and about 60% of an administered dose of salicylate itself is excreted as salicylate in cat urine.

Only tri-o-cresyl phosphate could be recovered from cat urine in measurable quantities after the intraperitoneal administration of TOCP. If salicylic acid, o-cresol, or o-hydroxybenzyl alcohol are metabolic products of TOCP, then they are excreted as salicylate in quantities too small for detection.

The intraperitoneal administration of 100 mg/kg of tri-o-cresyl phosphate in Tween 80 to cats seems to provide a reservoir of slowly metabolized TOCP. These studies do not rule out the possibility that o-cresol, o-hydroxybenzyl alcohol and salicylate are metabolic products of TOCP but if they are formed the quantities which are converted to salicylate cannot be distinguished from normal control levels of urinary salicylate.

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